Oxygen surrogate systems for supporting human drug-metabolizing cytochrome P450 enzymes

Silja J Strohmaier, James J De Voss, Ulrik Jurva, Shalini Andersson, Elizabeth M J Gillam

School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Australia (SJS, JJDV, EMJG)

DMPK, Early Cardiovascular, Renal and Metabolism, BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden (UJ)

Discovery Sciences, BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden (SA)

Running title: Oxygen surrogate systems for major drug-metabolizing P450s

Correspondence should be addressed to:

Elizabeth M.J. Gillam, School of Chemistry and Molecular Biosciences, The University of Queensland, St. Lucia, Brisbane, 4072, Australia

Tel: +61-7-3365 1410 Email: e.gillam@uq.edu.au

Web: https://staff.scmb.uq.edu.au/staff/elizabeth-gillam

Number of text pages: 25

Number of tables: 0

Number of figures: 2

Number of references: 58

Number of words in *Abstract*: 206

Number of words in *Introduction*: 674

Number of words in *Results and Discussion*: 1102

Non-standard abbreviations: BAIB, (diacetoxyiodo)benzene; CPR, cytochrome P450 reductase; CuOOH, cumene hydroperoxide; F-BAIB, bis(trifluoroacetoxy)iodobenzene; hCPR, human NADPH-cytochrome P450 reductase; LSF, late stage functionalization; luciferin H-EGE, ethylene glycol ester of 6'-desoxyluciferin; luciferin ME-EGE, ethylene glycol ester of luciferin 6'-methyl ether; luciferin MultiCYP, methyl ester of luciferin 6'-methyl ether; NGS, NADPH-generating system; OS, oxygen surrogate; P450, cytochrome P450, heme-thiolate protein P450; PhIO, iodosylbenzene; *tert*-BuOOH , tert-butyl hydroperoxide.

DMD Fast Forward. Published on April 1, 2020 as DOI: 10.1124/dmd.120.090555
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Oxygen surrogates (OSs) have been used to support cytochrome P450 enzymes for diverse purposes in drug metabolism research, including reaction phenotyping, mechanistic and inhibition studies, studies of redox partner interactions, and to avoid the need for NADPH or a redox partner. They also have been used in engineering P450s for more cost-effective, NADPH-independent biocatalysis. However, despite their broad application little is known of the preference of individual P450s for different OSs, or the substrate dependence of OS-supported activity. Furthermore, the biocatalytic potential of OSs other than cumene hydroperoxide (CuOOH) and hydrogen peroxide (H_2O_2) is yet to be explored. Here, we investigated the ability of the major human drug-metabolizing P450s, namely CYP3A4, CYP2C9, CYP2C19, CYP2D6 and CYP1A2, to use different OSs: H_2O_2; tert-butyl hydroperoxide (*tert*-BuOOH); CuOOH; (diacetoxyiodo)benzene (BAIB); and bis(trifluoroacetoxy)iodobenzene (F-BAIB). Overall, CuOOH and *tert*-BuOOH were found to be the most effective at supporting these P450s. However, the ability of P450s to be supported by OSs effectively was also found to be highly dependent on the substrate used. This suggests that the choice of OS should be tailored to both the P450 and the substrate under investigation, underscoring the need to employ screening methods that reflect the activity towards the substrate of interest to the end application.

DMD Fast Forward. Published on April 1, 2020 as DOI: 10.1124/dmd.120.090555
This article has not been copyedited and formatted. The final version may differ from this version.

Significance statement

Cytochrome P450 enzymes can be supported by different oxygen surrogates (OSs), avoiding the need for a redox partner and costly NADPH. However few data exist comparing relative activity with different OSs and substrates. This study shows that the choice of OS used to support the major drug-metabolizing P450s influences their relative activity and regioselectivity in a substrate specific fashion, and provides a model for the more efficient use of P450s for metabolite biosynthesis.

Introduction

Cytochrome P450 enzymes (P450s) are a superfamily of heme-containing monooxygenases capable of mediating a variety of chemically challenging reactions on a diverse set of substrates (Guengerich, 2001; Guengerich, 2005; Hrycay and Bandiera, 2008; de Montellano, 2015; Hrycay and Bandiera, 2015). The human xenobiotic-metabolizing P450 enzymes are collectively responsible for 80-90% of human oxidative metabolism of xenobiotics, with the major contributors being CYP3A4 (30.2%), CYP2D6 (20%), CYP1A2 (8.9%), CYP2C19 (6.8%), and CYP2C9 (12.8%) (Zanger and Schwab, 2013). Therefore, these enzymes have been the focus of efforts to determine which forms metabolize new drugs (reaction phenotyping) and the prediction of drug-drug interactions (Zanger and Schwab, 2013). Furthermore, due to their ability to act on a remarkably broad set of substrates in an often highly stereo-, chemo- and regio-selective manner, human drug-metabolizing P450s have been used as biocatalysts for the production of authentic drug metabolites (Vail et al., 2005). More recently, they have been investigated for late stage functionalization (LSF) of existing drug scaffolds (Obach et al., 2018; Fessner, 2019) and other fine chemicals. However, their direct application in metabolite production and LSF is hindered by their often low catalytic activity and the need for a redox partner, typically cytochrome P450 reductase (CPR), an expensive cofactor (NADPH) which limits the economic feasibility of P450-dependent processes (Girhard et al., 2015). While whole cell biocatalysts and *in vitro* cofactor regenerating systems can address the cost of cofactor provision to some degree, whole cell systems rely on the substrate passing through the cell wall and the reaction mixture is more complex in both cases, all of which limits subsequent product recovery.

Oxygen surrogates (OSs), such as peroxides and hypervalent iodine compounds have been known for many years to support P450 activity in the absence of a redox partner and cofactor, by virtue of the peroxide shunt in the P450 catalytic cycle or through direct transfer of an

oxygen atom to the P450 ferric heme (Hrycay and O'Brien, 1971b; Kadlubar et al., 1973; Cho et al., 2007).Various OSs have been identified to support P450 catalysis (Hrycay and O'Brien, 1971a; Hrycay et al., 1972; Kadlubar et al., 1973; Chance et al., 1979; Tan et al., 1983; Lindstrom and Aust, 1984; Hecker et al., 1987; Weiss et al., 1987; Vaz et al., 1990; Plastaras et al., 2000) including most commonly, CuOOH, *tert*-BuOOH, H_2O_2 and iodosylbenzene (PhIO). They have been particularly used in investigations of the interactions of P450s with their redox partners concerning potential allosteric effects, the mechanisms of P450 inhibition, the exploration of the P450-redox partner interaction surface and other mechanistic questions (Guengerich et al., 1997; Keizers et al., 2005; Lin et al., 2012; Varfaj et al., 2014; Yoshimoto et al., 2016).

While the use of OSs in P450 research is well established, their application in biocatalysis has attracted attention more recently. Implementation of OSs could make P450 catalysis more economically viable, but an inherent drawback in the use of OSs is the rapid inactivation of the P450 in the presence of peroxides and other reactive OSs, combined with the typically low efficiency of OS-mediated monooxygenation reactions (He et al., 1998; Kumar et al., 2005; Kumar et al., 2006). Therefore, several studies have attempted to improve the ability of P450s to use cheap OSs by directed evolution or rational engineering approaches (Joo et al., 1999; Cirino and Arnold, 2002; Cirino and Arnold, 2003; Keizers et al., 2005; Kumar et al., 2005; Kumar et al., 2006; Otey et al., 2006). More recent studies have aimed to optimize conditions for using peroxides to support P450 activities, but amongst human drug-metabolizing P450s, only CYP3A4 and CYP2D6 have been studied in any detail. No reports have examined iodosylbenzene derivatives for biocatalytic applications with drug-metabolizing P450s (Chefson et al., 2006; Kumar et al., 2006) although they have been explored with microbial forms (Dangi et al., 2018). Therefore, we set out to perform a more systematic analysis of the five major human xenobiotic-metabolizing P450s with several different commonly available

OSs to determine the most appropriate OS to use for each form for both mechanistic studies and biocatalytic applications.

Methods and Materials

Materials

The pCW vector from which all expression plasmids were derived was provided by Prof. F. W. Dahlquist (University of Oregon, Eugene, OR, USA). The bicistronic expression constructs pCW/CYP1A2/hCPR, pCW/CYP2C9$_{FL}^{His}$/hCPR, pCW/2C19$_{FL}^{His}$/hCPR, pCW/2D6 (Variant DB11) and pCW/CYP3A4/hCPR were constructed as described previously (Sandhu et al., 1994; Gillam et al., 1995; Richardson et al., 1995; Parikh et al., 1997; Shukla et al., 2005). For all P450s except CYP2D6, these plasmids encode an enzyme modified to encode the peptide 'MALLLAVFL' in the N-terminal in place of the native hydrophobic anchor to enable expression in bacteria. For CYP2D6, the N-terminus was replaced in the expressed enzyme with the peptide 'MARQVHSSWNL' upstream of the proline-rich region that marks the start of the catalytic domain. DH5aF'IQ strain cells were obtained from Sigma Aldrich and were pre-transformed with the pGro7 plasmid which was obtained from Dr. H. Yanagi (HSP Research Institute, Shimogyo-ku, Kyoto, Japan; Nishihara et al., 1998).

Expression of P450s

Expression of recombinant P450s in *E. coli* and preparation of subcellular bacterial membrane fractions was performed according to established methods (Gillam et al., 1993). P450 concentrations were measured via Fe(II)-CO versus Fe(II) difference spectroscopy and hCPR concentrations were quantified using the cyt *c* reduction assay, both as performed as described by Guengerich et al. (Guengerich et al., 2009).

Enzymatic assays

All assays were conducted with bacterial membrane fractions containing 0.5 µM of P450 co-expressed with hCPR in 100 mM potassium phosphate buffer (pH 7.4). Reactions were initiated by the respective support system, i.e., OS or an NADPH-regenerating system (NGS; final concentrations: 100 mM potassium phosphate buffer (pH 7.4), 0.25 mM $NADP^+$, 10 mM glucose-6-phosphate, 0.5 units/mL of glucose-6-phosphate dehydrogenase). Final concentrations of OSs were 1 mM, 0.5 mM, 0.25 mM and 0.1 mM. All reactions were incubated at 37 °C for the times indicated in descriptions of individual assays below, with moderate shaking to allow aeration of samples. As a control, reactions were carried out in parallel in the absence of enzyme, which resulted in no detectable product formation in all cases. All incubations were performed according to a preset plan with three independent replicates. Except for alkoxyresorufins, which were monitored continuously, reactions for all substrates were carried out at a single, high substrate concentration and terminated at a fixed time, since our purpose was to screen for general ability to use OSs to detect broad differences between P450s rather than to compare kinetics.

Dealkylation or hydroxylation of the luminogenic substrates was measured in opaque, white 384-well plates with the luciferin derivatives at the concentrations recommended by the manufacturer (50 µM luciferin MultiCYP, 6 µM luciferin 1A2, 100 µM luciferin-H 30 µM luciferin-ME-EGE, 10 µM luciferin-H EGE and 50 µM luciferin PFBE; Promega, Madison, WI). The 20 µl incubations were quenched after 60 mins by the addition of an equal volume of the respective luminescence detection reagent. Samples were left for 20 minutes at 25 °C to allow the development of luminescence before analysis in a luminescence detector (CLARIOstar® Plus Multi-Mode Microplate Reader, Ortenberg, BMG Labtech, Germany). For assays with luminogenic substrates, the luciferin detection reagent contained luciferase (all substrates) (luciferin ME-EGE, luciferin MultiCYP, esterase (luciferin ME-EGE, luciferin ME-EGE, luciferin H-EGE) and D-cysteine (luciferin 1A2) so the tolerance of the luciferase

reaction towards applied OSs was assessed at 1 mM of each OS. The tolerance of the esterase and luciferase in the detection reagent to OSs was tested by carrying out a CYP2D6-dependent reaction supported by NADPH/hCPR, as described above, using luciferin-ME-EGE as the substrate. The reaction, however, was quenched by adding an equal volume of 2 mM OS. Subsequently, an equal volume (2 volumes of the reaction volume) of detection reagent was added. The result was compared to a control reaction to which 0.5 volume of 100 mM potassium phosphate buffer (pH 7.4) was added rather than OS. The same procedure was applied to test the tolerance of D-cysteine towards OSs but using CYP1A2 and the respective substrate (luciferin-1A2) and detection reagent.

The *O*-dealkylation of 7-methoxyresorufin (5 µM) was measured in a continuous assay using a Spectramax M2 microplate reader (Molecular Devices, Sunnyvale, CA, USA), as described (Anari et al., 1997). Resorufin fluorescence was measured every five seconds over a total time of 30 mins at excitation and emission wavelengths of 530 nm and 582 nm respectively. Initial rates were determined from the linear range with reference to a resorufin standard.

For the metabolism of diclofenac, the assay was performed as described previously with slight modifications (Strohmaier et al.). Briefly, reactions were carried out in potassium phosphate buffer (100 mM, pH 7.4) at 37 °C, using 200 µM diclofenac in a final reaction volume of 200 µl and were initiated with the respective support system as described above. Reactions were terminated after 60 mins by transferring 200 µl to a tube containing 50 µl of acetic acetonitrile (5.66% (v/v) acetic acid in acetonitrile) and 10 µl of 1 mM *p*-nitrocatechol (internal standard) and were processed following the same procedure as described previously (Strohmaier et al.).

Caffeine metabolism and testosterone metabolism were carried out as described previously using 100 µM of the respective substrate (Strohmaier et al.).

Assays with omeprazole were conducted in the dark with an initial concentration of 100 µM omeprazole, in a total reaction volume of 250 µl. The reactions were quenched after 60 mins

by addition of the internal standard (25 µl of 1 mM progesterone), and thorough mixing with 1 mL ethyl acetate for one minute. After 10 min centrifugation at 18000 x *g*, 800 µl of the organic phase were transferred to a clean tube and evaporated to dryness under nitrogen. The dried extracts were resuspended in 24 µl acetonitrile, followed by the addition of 76 µl highly purified water immediately prior to analysis. Samples were analyzed using a 150 mm x 5 µM C18 column, (Agilent, Santa Clara, California, U.S.A) at a flow rate of 1.5 mL/min using a gradient from 24% to 63% acetonitrile in water over 18 min. Metabolites were detected by absorbance at 302 nm.

The hydroxylation of midazolam was carried out with 300 µM midazolam in a total reaction volume of 250 µl. After 60 minutes, 25 µl of 1 mM nordazepam were added and the reactions were quenched by the addition of 1 mL ethyl acetate with subsequent extraction followed by phase separation by centrifugation at 18 000 x *g* for 10 min. HPLC analysis was conducted as described previously (Hunter et al., 2011).

Dextromethorphan metabolism was assayed at 100 µM. Reactions were terminated after 60 mins by the addition of 25 µl of 1 mM *p*-nitrocatechol and thorough mixing with 1 mL ethyl acetate followed by phase separation at 18 000 x *g* for 10 min. Subsequent analysis was performed according to a previously described method (Gumulya et al., 2019).

Results and Discussion

Five commercially available, inexpensive OSs were studied here: H_2O_2; *tert*-BuOOH; CuOOH; BAIB; and F-BAIB. Overall, organic hydroperoxides were most effective at supporting P450-mediated activity, while much lower activity, if any, was observed with H_2O_2 or the iodosylbenzene derivatives studied (Figure 1). This may be due to rapid inactivation of the P450s by such OSs, as observed in other studies (Gustafsson et al., 1979; He et al., 1998; Yoshimoto et al., 2016; Albertolle et al., 2017). The optimal concentration of OS for supporting

each P450 was consistent across multiple substrates. Generally, product formation was highest with the lower concentrations of CuOOH, either 0.1 mM (CYP2C19) or 0.25 mM CuOOH (CYP1A2, CYP2C9 and CYP2D6), but CYP3A4 showed a slight increase in metabolite formation at 0.5 and 1 mM. By contrast, *tert*-BuOOH-supported activity was maximal at the highest concentration tested here in all cases, suggesting a less effective association between the ferric heme and *tert*-BuOOH and/or enhanced resistance of the P450s towards oxidative damage by *tert*-BuOOH compared to CuOOH.

The activities of CYP3A4 and CYP2D6 supported by *tert*-BuOOH and CuOOH in our study were lower relative to the NADPH-supported control than those reported previously by Chefson et al. (Chefson et al., 2006). One key difference between the present and previous studies was that NADPH was used directly in Chefson et al. (Chefson et al., 2006) whereas an NADPH-regenerating system was used here. NADPH is usually rapidly converted in P450 reactions to $NADP^+$, which can inhibit CPR. Thus, this may have led to lower activity of the control in the study reported by Chefson et al. (Chefson et al., 2006) Alternatively, differences in the P450:CPR ratio or other incubation conditions between our and the earlier study may have caused the discrepancy.

In the present study, hydrogen peroxide was the least effective OS overall which agrees with several other studies on microsomal P450s reported previously (Kadlubar et al., 1973; Kumar et al., 2005; Chefson et al., 2006). CYP1A2-mediated 7-methoxyresorufin *O*-demethylase activity in liver microsomes was reported to be supported effectively by H_2O_2 (Anari et al., 1997); however, we were unable to confirm this result using recombinant human CYP1A2 (Supplementary Figure 1). CYP3A4-dependent N-oxide formation from quinidine was reported to be comparable between reactions supported by H_2O_2 and NADPH/CPR/O_2 (Guengerich and Johnson, 1997). Similarly, CYP2B1 and CYP2B4 metabolized *N*,*N*-dimethylaniline to the corresponding N-oxide equally effectively in the presence of H_2O_2 as in

reactions supported by NADPH (Guengerich and Johnson, 1997). Collectively, this suggests that the ability of H_2O_2 to support activity may depend to some extent on the type of the reaction. Notably, no N-oxidation reactions were included amongst the activities studied in this work.

For most substrates only a single significant metabolite could be detected (as shown in Figure 1) however for caffeine metabolism by CYP1A2 and midazolam metabolism by CYP3A4, multiple products were detected at measurable levels (Figure 2). (We cannot exclude the possibility that additional metabolites may have been formed from alkoxyresorufins and luciferin derivatives, but to our knowledge, there is no precedent for alternative metabolites not detectable by the methods used.) The regioselectivity of midazolam and caffeine metabolism was altered slightly but significantly when using organic peroxides compared to NADPH/CPR-supported reactions (Figure 2, Supplementary Tables 1 and 2). Similar observations have been made previously with various P450s and diverse substrates (Hrycay et al., 1975; Hanna et al., 2001; Yoshimoto et al., 2016; Dangi et al., 2018). This phenomenon is generally explained by a greater tendency of these OSs to undergo homolytic O-O bond scission as compared to H_2O_2, due to electron donating substituents (White and Coon, 1980; Barr et al., 1996; Nam et al., 2000). This may result in a mechanism in which hydrogen abstraction from the substrate is mediated by the resulting alkoxy radical rather than by compound I (White and Coon, 1980; Blake and Coon, 1981; Weiss and Estabrook, 1986). Therefore, the changes in regioselectivity observed may be related to the different positioning of the alkoxy radical and compound I relative to the substrate.

Overall, for each isoform, the relative activity supported by the OSs differed according to which substrate was used to evaluate activity. This phenomenon was particularly pronounced in case of *tert*-BuOOH-supported metabolism. This observation that relative activity differs in a substrate-dependent fashion is consistent with a previous study in which the K_M ^{CuOOH} of

CYP3A4 differed with different substrates (Kumar et al., 2006). The authors speculated that the active site could be masked to a greater or lesser extent, depending on the substrate, hampering access of the OS to the heme resulting in substrate-dependent changes of the K_M^{CuOOH}. In such a scenario, a sterically less complex, albeit hydrophobic OS (i.e. *tert*-BuOOH), would have better access to the active site than a more larger one (i.e., CuOOH). If accessibility of the OS was diminished by a larger hydroperoxide, one might expect that the relative activity with e.g., CuOOH, would be affected to a larger extent than that supported by a simpler peroxide (e.g. tert-BuOOH) in the presence of different substrates. If hydrogen abstraction from the substrate is mediated by the resulting alkoxy radical rather than by compound I, the orientations of the substrate and OS relative to the heme in the active site may determine the relative effectiveness of hydrogen abstraction by the OS-derived alkoxy radical and subsequent substrate monooxygenation by oxygen rebound from the heme. No metabolite formation was observed for any of the OSs in the absence of a P450, ruling out the possibility that purely chemical reactions contribute to the differences seen.

We also considered whether the remnant of the OS may have affected the orientation or accessibility of the substrate to the heme, leading to increased or decreased turnover. However, addition of 1mM *tert*-butanol to the NADPH/CPR-supported incubations of CYP3A4 with testosterone and midazolam, affected neither product formation nor stereoselectivity (data not shown), suggesting that such an effect is unlikely.

In conclusion, this study has revealed that the organic peroxides, CuOOH and *tert*-BuOOH, are the best starting points for supporting activity of the major drug-metabolizing P450s in the absence of NADPH, molecular oxygen or a redox partner, but that the relative activity supported by OSs is substrate-dependent. Further studies will be necessary to explore the nature of this phenomenon. The potential for alterations in regioselectivity of substrate oxidation should be considered when OSs are used for mechanistic studies. Likewise, when OSs are used

to support the activity of P450s for biocatalysis, the OS used should be tailored to both the P450 and the substrate under investigation, underscoring the need to employ screening methods that reflect the activity towards the substrate of interest to the end application.

Authorship contributions

Participated in research design: Strohmaier, Gillam

Conducted experiments: Strohmaier

Contributed new reagents or analytic tools: De Voss, Jurva, Andersson

Performed data analysis: Strohmaier, Gillam

Wrote or contributed to the writing of the paper: Strohmaier, De Voss, Jurva, Andersson, Gillam

References

Albertolle ME, Kim D, Nagy LD, Yun C-H, Pozzi A, Savas Ü, Johnson EF, and Guengerich FP (2017) Heme–thiolate sulfenylation of human cytochrome P450 4A11 functions as a redox switch for catalytic inhibition. *J Biol Chem* **292:**11230-11242.

Anari MR, Josephy PD, Henry T, and O'Brien PJ (1997) Hydrogen peroxide supports human and rat cytochrome P450 1A2-catalyzed 2-amino-3-methylimidazo[4,5-f]quinoline bioactivation to mutagenic metabolites: significance of cytochrome P450 peroxygenase. *Chem Res Toxicol* **10:**582-588.

Barr DP, Martin MV, Guengerich FP, and Mason RP (1996) Reaction of cytochrome P450 with cumene hydroperoxide: ESR spin-trapping evidence for the homolytic scission

of the peroxide O-O bond by ferric cytochrome P450 1A2. *Chem Res Toxicol* **9:**318-325.

Blake RC, II and Coon MJ (1981) On the mechanism of action of cytochrome P-450: Evaluation of homolytic and heterolytic mechanisms of oxygen-oxygen bond cleavage during substrate hydroxylation by peroxides. *J Biol Chem* **256:**12127-12133.

Chance B, Sies H, and Boveris A (1979) Hydroperoxide metabolism in mammalian organs. *Physiol Rev* **59:**527-605.

Chefson A, Zhao J, and Auclair K (2006) Replacement of the natural cofactors by selected hydrogen peroxide donors or organic peroxides results in improved activity for CYP3A4 and CYP2D6. *ChemBioChem* **7:**916-919.

Cho KB, Moreau Y, Kumar D, Rock DA, Jones JP, and Shaik S (2007) Formation of the active species of cytochrome P450 by using iodosylbenzene: a case for spin-selective reactivity. *Chemistry* **13:**4103-4115.

Cirino PC and Arnold FH (2002) Regioselectivity and activity of cytochrome P450 BM-3 and mutant F87A in reactions driven by hydrogen peroxide. *Adv Synth Catal* **344:**932-937.

Cirino PC and Arnold FH (2003) A self-sufficient peroxide-driven hydroxylation biocatalyst. *Angew Chem Int Ed Engl* **42:**3299-3301.

Dangi B, Park H, and Oh TJ (2018) Effects of alternative redox partners and oxidizing agents on CYP154C8 catalytic activity and product distribution. *ChemBioChem* **19:**2273-2282.

de Montellano PRO (2015) Substrate oxidation by cytochrome P450 enzymes, in: *Cytochrome P450; Structure, Mechanism, and Biochemistry* (Ortiz de Montellano PR ed), pp 111-176, Springer.

Fessner ND (2019) P450 Monooxygenases enable rapid late-stage diversification of natural products via C-H bond activation. *ChemCatChem* **11:**2226-2242.

Gillam EMJ, Baba T, Kim B-R, Ohmori S, and Guengerich FP (1993) Expression of modified human cytochrome P450 3A4 in *Escherichia coli* and purification and reconstitution of the enzyme. *Arch Biochem Biophys* **305:**123-131.

Gillam EMJ, Guo ZY, Martin MV, Jenkins CM, and Guengerich FP (1995) Expression of cytochrome P450 2D6 in *Escherichia coli:* Purification, and spectral and catalytic characterization. *Arch Biochem Biophys* **319:**540-550.

Girhard M, Bakkes PJ, Mahmoud O, and Urlacher VB (2015) P450 biotechnology, in: *Cytochrome P450; Structure, Mechanism, and Biochemistry* (Ortiz de Montellano PR ed), pp 451-520, Springer International Publishing, Cham.

Guengerich FP (2001) Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity. *Chem Res Toxicol* **14:**611-650.

Guengerich FP (2005) Human cytochrome P450 enzymes, in: *Cytochrome P450* (Ortiz de Montellano PR ed), pp 377-530, Kluwer Academic/Plenum Publishing, New York.

Guengerich FP and Johnson WW (1997) Kinetics of ferric cytochrome P450 reduction by NADPH-cytochrome P450 reductase: Rapid reduction in the absence of substrate and variations among cytochrome P450 systems. *Biochemistry* **36:**14741-14750.

Guengerich FP, Martin MV, Sohl CD, and Cheng Q (2009) Measurement of cytochrome P450 and NADPH-cytochrome P450 reductase. *Nat Protocols* **4:**1245-1251.

Guengerich FP, Vaz AD, Raner GN, Pernecky SJ, and Coon MJ (1997) Evidence for a role of a perferryl-oxygen complex, FeO^{3+}, in the N-oxygenation of amines by cytochrome P450 enzymes. *Mol Pharmacol* **51:**147-151.

Gumulya Y, Huang W, D'Cunha SA, Richards KE, Thomson RES, Hunter DJB, Baek J-M, Harris KL, Boden M, De Voss JJ, Hayes MA, Isin EM, Andersson S, Jurva U, and

Gillam EMJ (2019) Engineering thermostable CYP2D enzymes for biocatalysis using combinatorial libraries of ancestors for directed evolution (CLADE). *ChemCatChem* **11:**841-850.

Gustafsson JÅ, Rondahl L, and Bergman J (1979) Iodosylbenzene derivatives as oxygen donors in cytochrome P-450 catalyzed steroid hydroxylations. *Biochemistry* **18:**865-870.

Hanna IH, Krauser JA, Cai H, Kim M-S, and Guengerich FP (2001) Diversity in mechanisms of substrate oxidation by cytochrome P450 2D6: lack of an allosteric role of NADPH-cytochrome P450 reductase in catalytic regioselectivity. *J Biol Chem* **276:**39553-39561.

He K, Bornheim LM, Falick AM, Maltby D, Yin HQ, and Correia MA (1998) Identification of the heme-modified peptides from cumene hydroperoxide-inactivated cytochrome P450 3A4. *Biochemistry* **37:**17448-17457.

Hecker M, Baader WJ, Weber P, and Ullrich V (1987) Thromboxane synthase catalyses hydroxylations of prostaglandin H2 analogs in the presence of iodosylbenzene. *Eur J Biochem* **169:**563-569.

Hrycay EG and Bandiera SM (2008) Cytochrome P450 enzymes. *Preclinical development handbook: ADME and biopharmaceutical properties Wiley, Hoboken***:**627-696.

Hrycay EG and Bandiera SM (2015) Monooxygenase, peroxidase and peroxygenase properties and reaction mechanisms of cytochrome P450 enzymes, in: *Monooxygenase, Peroxidase and Peroxygenase Properties and Mechanisms of Cytochrome P450* (Hrycay EG and Bandiera SM eds), pp 1-61, Springer International Publishing, Cham.

Hrycay EG, Gustafsson JA, Ingelman-Sundberg M, and Ernster L (1975) Sodium periodate, sodium-chlorite, organic hydroperoxides, and H2O2 as hydroxylating agents in

steroid hydroxylation reactions catalyzed by partially purified cytochrome-P-450 *Biochem Biophys Res Comm* **66:**209-216.

Hrycay EG and O'Brien PJ (1971a) Cytochrome P-450 as a microsomal peroxidase utilizing a lipid peroxide substrate. *Arch Biochem Biophys* **147:**14-27.

Hrycay EG and O'Brien PJ (1971b) The peroxidase nature of cytochrome P-420 utilizing a lipid peroxide substrate. *Arch Biochem Biophys* **147:**28-35.

Hrycay EG, O'Brien PJ, Van Lier JE, and Kan G (1972) Pregnene 17α-hydroperoxides as possible precursors of the adrenosteroid hormones. *Arch Biochem Biophys* **153:**495-501.

Hunter DJB, Behrendorff JBYH, Johnston WA, Hayes PY, Huang W, Bonn B, Hayes MA, De Voss JJ, and Gillam EMJ (2011) Facile production of minor metabolites for drug development using a CYP3A shuffled library. *Metab Eng* **13:**682-693.

Joo H, Lin Z, and Arnold FH (1999) Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation. *Nature* **399:**670-673.

Kadlubar FF, Morton KC, and Ziegler DM (1973) Microsomal-catalyzed hydroperoxide-dependent C-oxidation of amines. *Biochem Biophys Res Comm* **54:**1255-1261.

Keizers PH, Schraven LH, de Graaf C, Hidestrand M, Ingelman-Sundberg M, van Dijk BR, Vermeulen NP, and Commandeur JN (2005) Role of the conserved threonine 309 in mechanism of oxidation by cytochrome P450 2D6. *Biochem Biophys Res Comm* **338:**1065-1074.

Kumar S, Chen CS, Waxman DJ, and Halpert JR (2005) Directed evolution of mammalian cytochrome P450 2B1. Mutations outside of the active site enhance the metabolism of several substrates, including the anticancer prodrugs cyclophosphamide and ifosfamide. *J Biol Chem* **280:**19569-19575.

Kumar S, Liu H, and Halpert JR (2006) Engineering of cytochrome P450 3A4 for enhanced peroxide-mediated substrate oxidation using directed evolution and site-directed mutagenesis. *Drug Metab Dispos* **34:**1958-1965.

Lin HL, Kenaan C, Zhang HM, and Hollenberg PF (2012) Reaction of human cytochrome P450 3A4 with peroxynitrite: nitrotyrosine formation on the proximal side impairs its interaction with NADPH-cytochrome P450 reductase. *Chem Res Toxicol* **25:**2642-2653.

Lindstrom TD and Aust SD (1984) Studies on cytochrome P-450-dependent lipid hydroperoxide reduction. *Arch Biochem Biophys* **233:**80-87.

Nam W, Han HJ, Oh S-Y, Lee YJ, Choi M-H, Han S-Y, Kim C, Woo SK, and Shin W (2000) New insights into the mechanisms of O−O Bond cleavage of hydrogen peroxide and tert-alkyl hydroperoxides by iron(III) porphyrin complexes. *J Am Chem Soc* **122:**8677-8684.

Nishihara K, Kanemori M, Kitagawa M, Yanagi H, and Yura T (1998) Chaperone coexpression plasmids: Differential and synergistic roles of DnaK-DnaJ-GrpE and GroEL-GroES in assisting folding of an allergen of Japanese Cedar pollen, Cryj2, in Escherichia coli. *Appl Environ Microbiol* **64:**1694-1699.

Obach RS, Walker GS, Sharma R, Jenkinson S, Tran TP, and Stepan AF (2018) Lead diversification at the nanomole scale using liver microsomes and quantitative nuclear magnetic resonance spectroscopy: application to phosphodiesterase 2 inhibitors. *J Med Chem* **61:**3626-3640.

Otey CR, Bandara G, Lalonde J, Takahashi K, and Arnold FH (2006) Preparation of human metabolites of propranolol using laboratory-evolved bacterial cytochromes P450. *Biotechnol Bioeng* **93:**494-499.

Parikh A, Gillam EMJ, and Guengerich FP (1997) Drug metabolism by *Escherichia coli* expressing human cytochromes P450. *Nat Biotech* **15:**784-788.

Plastaras JP, Guengerich FP, Nebert DW, and Marnett LJ (2000) Xenobiotic-metabolizing cytochromes P450 convert prostaglandin endoperoxide to hydroxyheptadecatrienoic acid and the mutagen, malondialdehyde. *J Biol Chem* **275:**11784-11790.

Richardson TH, Jung F, Griffin KJ, Wester M, Raucy JL, Kemper B, Bornheim LM, Hassett C, Omiecinski CJ, and Johnson EF (1995) A universal approach to the expression of human and rabbit cytochrome P450s of the 2C subfamily in Escherichia coli. *Arch Biochem Biophys* **323:**87-96.

Sandhu P, Guo Z, Baba T, Martin MV, Tukey RH, and Guengerich FP (1994) Expression of modified human cytochrome P450 1A2 in *Escherichia coli*: stabilization, purification, spectral characterization, and catalytic activities of the enzyme. *Arch Biochem Biophys* **309:**168-177.

Shukla A, Gillam EM, Mitchell DJ, and Bernhardt PV (2005) Direct electrochemistry of enzymes from the cytochrome P450 2C family. *Electrochem Commun* **7:**437-442.

Strohmaier SJ, Huang W, Baek J-M, Hunter DJB, and Gillam EMJ (2019) Rational evolution of the cofactor-binding site of cytochrome P450 reductase yields variants with increased activity towards specific cytochrome P450 enzymes. *FEBS J* **286:**4473-4493.

Tan L, Hrycay EG, and Matsumoto K (1983) Synthesis and properties of the epimeric 6-hydroperoxyandrostenediones, new substrates/inhibitors of human placental aromatase. *J Steroid Biochem* **19:**1329-1338.

Vail RB, Homann MJ, Hanna I, and Zaks A (2005) Preparative synthesis of drug metabolites using human cytochrome P450s 3A4, 2C9 and 1A2 with NADPH-P450 reductase expressed in Escherichia coli. *J Indust Microbiol Biotech* **32:**67-74.

Varfaj F, Zulkifli SNA, Park H-G, Challinor VL, De Voss JJ, and Ortiz de Montellano PR (2014) Carbon-carbon bond cleavage in activation of the prodrug nabumetone. *Drug Metab Dispos* **42:**828.

Vaz A, Roberts ES, and Coon MJ (1990) Reductive beta-scission of the hydroperoxides of fatty acids and xenobiotics: role of alcohol-inducible cytochrome P-450. *Proc Natl Acad Sci USA* **87:**5499-5503.

Weiss RH, Arnold JL, and Estabrook RW (1987) Transformation of an arachidonic acid hydroperoxide into epoxyhydroxy and trihydroxy fatty acids by liver microsomal cytochrome P-450. *Arch Biochem Biophys* **252:**334-338.

Weiss RH and Estabrook RW (1986) The mechanism of cumene hydroperoxide-dependent lipid peroxidation: the function of cytochrome P-450. *Arch Biochem Biophys* **251:**348-360.

White RE and Coon MJ (1980) Oxygen activation by cytochrome P-450. *Annu Rev Biochem* **49:**315-356.

Yoshimoto FK, Gonzalez E, Auchus RJ, and Guengerich FP (2016) Mechanism of 17alpha,20-lyase and new hydroxylation reactions of human cytochrome P450 17A1: ^{18}O labeling and oxygen surrogate evidence for a role of a perferryl oxygen. *J Biol Chem* **291:**17143-17164.

Zanger UM and Schwab M (2013) Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacol Therap* **138:**103-141.

Footnotes

(unnumbered footnote to title)

This work was supported by an Australian Research Council Discovery Project Grant DP160100865 and by AstraZeneca Innovative Medicines and Early Development, Cardiovascular, Renal and Metabolism, Gothenburg. SJS was funded by an International Postgraduate Research Award from the University of Queensland.

(unnumbered footnote to conference presentation)

This work was presented in preliminary form at the 21st International Conference on Cytochrome P450 Biochemistry and Biophysics (Brisbane, Australia, June 2019) and Biotrans 2019 (Groningen, The Netherlands, July 2019).

Correspondence should be directed to Elizabeth Gillam, School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Australia: e.gillam@uq.edu.au.

Figure legends

Figure 1

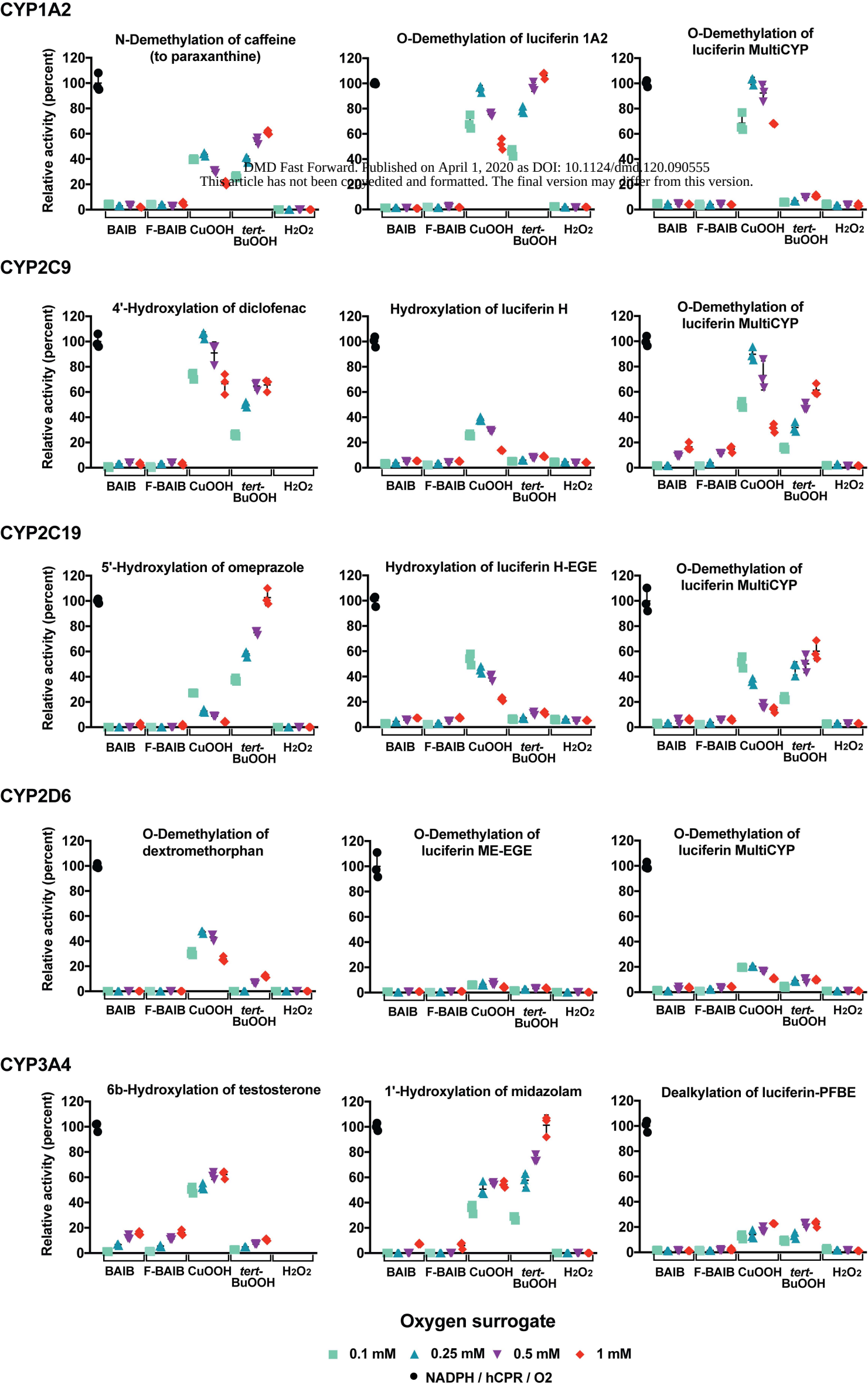
OSs support the activity of the major drug-metabolizing P450s in a substrate and OS-specific manner.

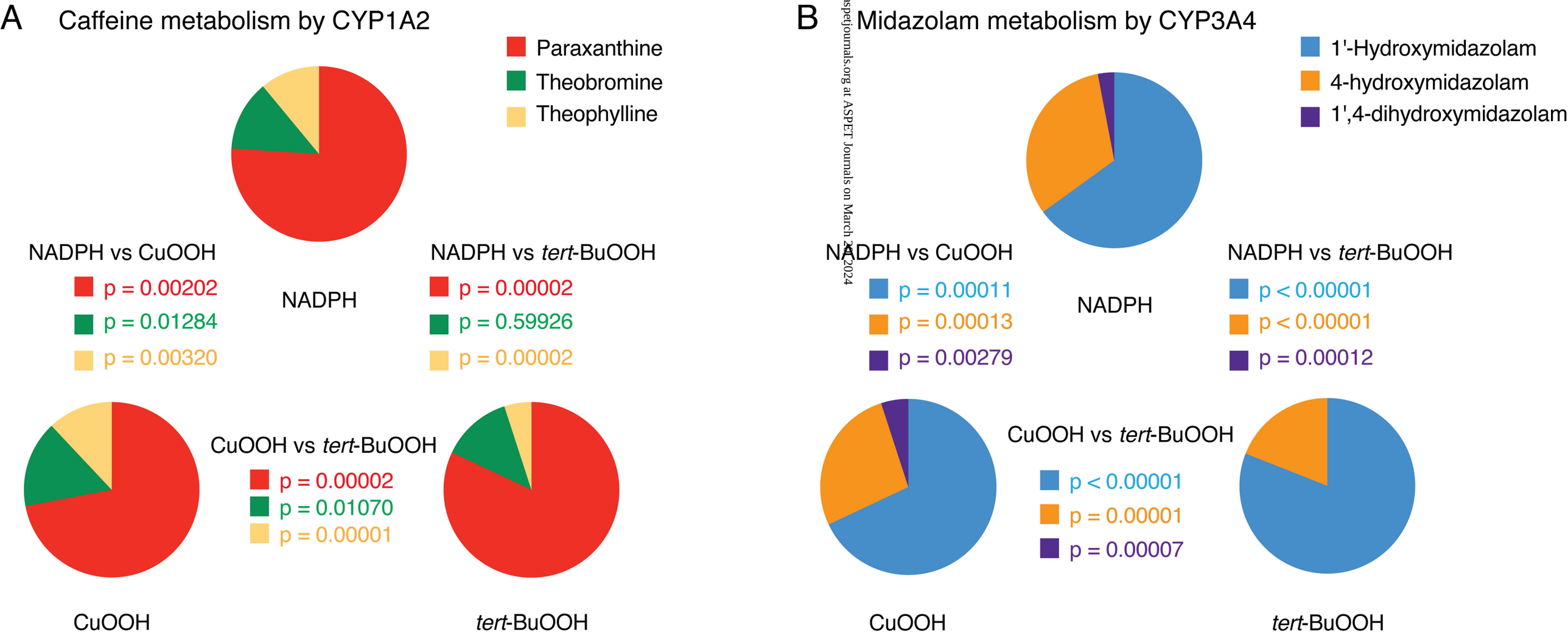
The major drug-metabolizing P450s were incubated with the substrates indicated in the presence of an NADPH-generating system (black circles) or one of four concentrations of each OS: 0.1 mM (light blue squares), 0.25 mM (blue triangles), 0.5 mM (purple inverted triangles) and 1 mM (red diamonds). Incubations were carried out for 60 mins and at the substrate concentrations as follow: 50 μM luciferin MultiCYP; 6 μM luciferin 1A2; 100 μM luciferin-H; 30 μM luciferin-ME-EGE; 10 μM luciferin-H EGE; 50 μM luciferin PFBE; 200 μM diclofenac; 100 μM caffeine; 100 μM testosterone; 100 μM omeprazole; 300 μM midazolam; and 100 μM dextromethorphan. Product formation is shown relative to the activity supported by hCPR and the NADPH generating system (set to 100%). Individual replicates are shown along with the means +/- SD of three replicate incubations.

Figure 2.

The regioselectivity of caffeine and midazolam metabolism by CYP1A2 and CYP3A4 is influenced by the support system used.

Pie charts show the relative formation of three metabolites of caffeine by CYP1A2 (A) or midazolam by CYP3A4 (B) in the presence of CuOOH, *tert*-BuOOH or an NADPH generating system, with oxygen and hCPR as indicated. Data represent the means of three independent incubations. P values indicate statistical significance (two-tailed unpaired Student's *t* test).





Supplementary information for:

Oxygen surrogate systems for supporting human drug-metabolizing cytochrome P450 enzymes

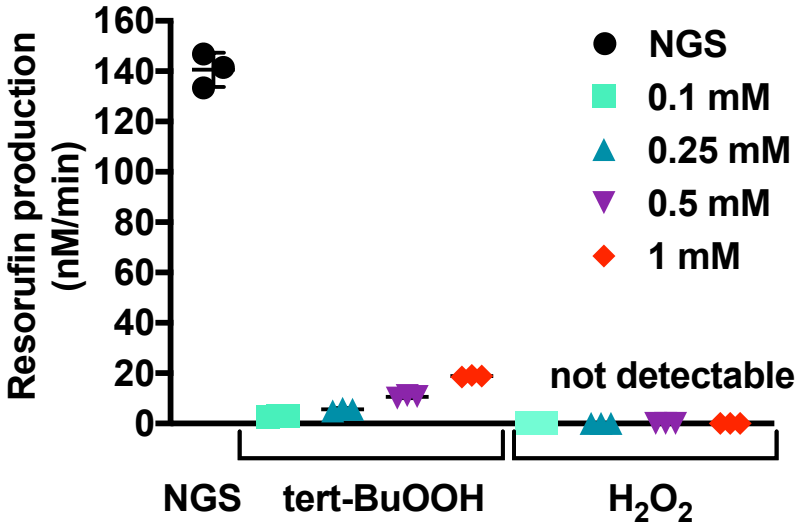
Silja J Strohmaier, James J De Voss, Ulrik Jurva, Shalini Andersson, Elizabeth M J Gillam

School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane, Australia (SJS, JJDV, EMJG)

DMPK, Early Cardiovascular, Renal and Metabolism, BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden (UJ)

Discovery Sciences, BioPharmaceuticals R&D, AstraZeneca, Gothenburg, Sweden (SA)

Supplementary information



Supplementary Figure 1

CYP1A2 activity can be supported by *tert*-BuOOH but not hydrogen peroxide.

CYP1A2 was incubated with 5 µM 7-methoxyresorufin in the presence of either an NADPH-generating system, *tert*-BuOOH or hydrogen peroxide. Individual replicates are shown along with the means +/- SD of three replicate incubations.

Supplementary Table 1

Regioselectivity of caffeine metabolism by CYP1A2

Metabolite[a]	NGS[b]	CuOOH	*tert*-BuOOH
Paraxanthine	75.7 ± 0.4 %	72.0 ± 0.8 %	82.4 ± 0.2 %
Theobromine[c,d]	13.2 ± 0.3 %[c,d]	15.6 ± 0.9 %	13.0 ± 0.4 %
Theophylline	11.1 ± 0.2 %	12.3 ± 0.3 %	4.5 ± 0.4 %

[a] Data for each metabolite and support system are all significantly different to the corresponding data for the same metabolite and each of the other support systems at the $p<0.005$ level or better (two-tailed unpaired Student's *t*-test) with the exception of theobromine production by CYP1A2.

[b] Reactions were supported by an NADPH-generating system (NGS) and used the coexpressed hCPR.

[c,d] Relative production of theobromine in both the NGS and *tert*-BuOOH-supported systems was significantly different from that in the CuOOH-supported system at the $p<0.05$ level, but there was no significant difference between the relative production of theobromine between the NGS and *tert*-BuOOH-supported systems.

Supplementary Table 2

Regioselectivity of midazolam metabolism by CYP3A4 [a]

Metabolite[a]	NGS	CuOOH	*tert*-BuOOH
1'-hydroxymidazolam	64.8 ± 0.4 %	67.9 ± 0.1 %	80.6 ± 0.1 %
4-hydroxymidazolam	32.2 ± 0.4 %	27.0 ± 0.5 %	19.0 ± 0.2 %
1',4-dihydroxymidazolam	3.0 ± 0.3 %	5.1 ± 0.5 %	0.4 ± 0.1 %

[a] Data for each metabolite and support system are all significantly different to the corresponding data for the same metabolite and each of the other support systems at the $p<0.005$ level or better (two-tailed unpaired Student's *t*-test).